Syringe method for stepwise chemical synthesis of oligonucleotides

Toshiki Tanaka and Robert L. Letsinger

Chemistry Department, Northwestern University, Evanston, IL 60201, USA

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ABSTRACT

A simple procedure is described for synthesis of oligonucleotides by phosphite chemistry. Chains can be constructed rapidly with minimal equipment (a syringe and reagent bottles). The method is illustrated by synthesis of d-TGCAGGTT. Pertinent supporting data on the effect of variations in the detritylation, condensation, oxidation, capping and cleavage steps in the synthetic approach and in isolation procedures are also presented.

INTRODUCTION

Adaptation of phosphite-triester chemistry¹ to solid phase syntheses conducted on silica gel has led to rapid procedures for the machine synthesis of defined oligonucleotides.^{2,3} Although highly attractive for use in laboratories where extensive synthetic work is done, the machines suffer the disadvantage of being expensive to buy or troublesome to construct. We describe herein a modification which permits one to conduct the repetitive synthetic steps rapidly with minimal equipment.

In principle, the operator sets up a series of small capped jars, each of which contains one of the solvents or reagents required in the synthesis. The silica support bearing a covalently attached nucleoside is placed in a syringe equipped with a filter at the base. Synthesis is carried out by successively drawing solutions in and expelling them from the syringe. The cycle is repeated for each nucleotide unit added to the chain; so the only operation required throughout the building step involves manipulation of the syringe. At the end of the cycles, the oligonucleotide is deprotected, removed from the silica, and isolated by previously described procedures. 2,3

Using this technique we examined a number of variables in the synthetic sequence. A simple procedure was developed which enables one to add specified nucleotide units to a growing chain rapidly. This procedure and the supporting study of reaction variables are reported in the RESULTS section.

METHODS AND MATERIALS

Equipment. Reagents were stored in Hypo-Vials (30 mL, Pierce Chem. Co.) closed with Hycar Septa. When samples were removed or added, pressure was equilized under nitrogen or by insertion of a small disposable syringe filled with a drying agent (Drierite). The reaction vessel was a glass syringe (~2 mL) fitted with a Luer lock and needle and with a filter, which was cut from porous polypropylene (~2 mm thick) and forced to the base of the barrel.

Thin layer chromatography was carried out with Whatman K5f silica and MKC $_{18}$ plates. HPLC was performed with a Perkin Elmer series 2 Liquid Chromatograph equipped with a Whatman Partisil PX10/25 ODS-2 column or PXS $_{10/25}$ SAX column.

Silica Support. Davidson 62 silica (140-200 mesh, 14 nm average pore diameter, 1.1 mL/g pore volume, $300 \text{ m}^2/\text{g}$ surface area) was derivatized as described by Matteuci and Caruthers³ with the exception that the succincylation step was carried out with succinic anhydride in anhydrous pyridine. The loading (determined by the absorbance of the dimethoxytrityl carbocation) amounted to 140 µmole thymidine per gram.

Solvents and Reagents. Tetrahydrofuran (THF) and pyridine were dried with calcium hydride, distilled, and stored over Molecular Sieves. The protected nucleosides, d-(DMTr)T, d-(DMTr)bzC, d-(DMTr)bzA, and d-(DMTr)ibG, were prepared by standard procedures. Methyl phosphorodichloridite (CH3OPCl₂)⁶ was distilled (bp 93-94°C; ³¹P NMR, 199 ppm relative to triphenyl phosphate and stored in a Hypo-Vial with an air tight septum.

Nucleoside Phosphorochloridite Reagents. The active nucleoside derivatives were prepared in an argon atmosphere in vials (10 mL) equipped with a magnetic stirring bar and closed by a septum. Reagents were added by syringe. Typically, the protected nucleoside (1 mmol) in THF (1 mL) was added dropwise over a 10 min period to a well stirred solution of CH3OPCl2 (1 mmol) in pyridine (0.4 mL) and THF (2 mL) at -78°C. After 5 min the mixture was warmed to room temperature (10 min) and centrifuged. The supernatant was transferred via syringe to a closed, well dried vial (40 mL) containing pyridine (2 mL); the precipitate was washed with THF (2 mL), and after centrifugation the supernatant was added to the vial containing the pyridine. The solvent was then stripped off under vacuum, leaving a foamy solid (the active nucleoside phosphorochloridite reagent). This material was taken up in pyridine (10 mL) for direct use in synthesis or storage at -70°C. These activated derivatives are stable for at least a

month at -70°C.

As a check on activity, an aliquot (10 μ L) of the pyridine solution may be quenched in CH₃OH² (20 μ L) and chromatographed on Whatman reverse phase plates (MKC₁₈F) with acetone-H₂O, 7:3. The dimethyl nucleoside phosphite (indicative of active phosphorochloridite reagent) is found as the major spot (~70% of trityl positive material) at R_f 0.25 to 0.35. Two additional spots are characteristically observed at R_f 0.09-0.22 (the 3'-3'-dinucleoside methyl phosphite) and at R_f 0.45-0.54 (ROPHOCH₃, formed by hydrolysis of the active reagent by advantitious water).

The reagents can also be checked conveniently by ³¹P NMR. CH₃OPCl₂, CH₃OP(Cl)OR, CH₃OP(OR)₂ and CH₃OP(O)H(OR) show peaks at -199, -185, -157, and -26 ppm respectively (relative to triphenyl phosphate). It is significant that the reagents prepared as described, with a single evaporation of THF and pyridine, are free of CH₃OPCl₂ within the detection limits of the NMR experiment.

RESULTS

Synthesis of d-TGCAGGTT. The general method is illustrated by a synthesis of d-TGCAGGTT. In addition to application of the syringe techniques novel features include elimination of a capping step in the cycle and use of nitromethane/methanol (99/1 v/v) as a solvent in cleaving dimethoxytrityl ethers with trichloroacetic acid.

Closed bottles containing the following solutions were prepared: (1) CH₃NO₂, (2) 3% Cl₃CCOOH in CH₃NO₂/CH₃OH (99/1 v/v), (3) CH₃NO₂ (4) dry pyridine, (5) nucleoside phosphorocloridite reagents, (6) 0.1 M iodine in THF/pyridine/H₂O, 40/20/1 v/v/v, (7) pyridine. A sample (36 mg) of silica loaded with 5'-O-dimethoxytritylthymidine (5 µmol) was placed in the syringe and synthesis was effected by drawing in and expelling solution from the syringe, proceeding stepwise from bottle 1-7. A schedule for each reaction cycle is given in Table 1.

The total time required for each cycle is about 13 min. In step 5, we found it convenient to rotate the syringe slowly mechanically during the 6 min reaction period, although manual rotation or shaking is adequate. For estimation of the coupling yields in the course of the synthesis, the absorbance at 500 nm ($\lambda_{\rm max}$ for the dimethoxytrityl cation in this medium) was determined. The ratio of the absorbance for the ion obtained from a given cycle to the absorbance obtained in the previous cycle is a measure of the efficiency of the condensation. By this test the average coupling

Table I. Schedule for One Cyc	cle in	Synthesis
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Ope:	ration	Solution	Volume
1.	Wash	CH ₃ NO ₂	1 mL (4 times)
2.	Detritylation	3% С1 ₃ ССООН	1 mL for 30 sec (5 times)
3.	Wash	CH ₃ NO ₂	1 mL (3 times)
4.	Wash	Pyridine	1 mL (5 times)
5.	Condensation	ROPC1(OCH ₃)	1 mL (6 min)
6.	Oxidation	0.1 M I ₂	1 mL (30 sec)
7.	Wash	Pyridine	1 mL (3 times)

yield was 94% and the overall yield was 61%.

For isolation of the oligonucleotide, the silica derivative was removed from the syringe and treated with 3 mL of a solution of $C_{6H_5SH/(C_2H_5)_3N/dioxane}$ (1/1/2 v/v/v) at room temperature for 30 min. It was then washed with CH₃OH (3 mL x 3) and H₂O (3 mL x 2) and treated with concd. NH₄OH (1 mL) at 50°C for 15 h. The NH₄OH solution was collected and the silica was washed with H₂O (2 mL x 2). The combined solutions were then lyophilized and the residue was taken up in H₂O (0.5 mL). Residual silica was separated by centrifugation.

For purification, a sample (22 $0D_{250}$ units, 8% of the total) of the crude material eluted from the solid support was separated by preparative TLC on a Brinkman silica plate (0.5 mm thick; 20 cm) by developing with n-C3H70H/NH40H/H20 (55/10/35 v/v/v). A well defined band corresponding to the octanucleotide (R_f 0.26) was the major band on the plate revealed under ultraviolet light. Elution with C_2H_50H/H_20 (1/3 v/v) evaporation, and dissolution in H_20 yielded a solution of d-TGCAGGTT, λ_{max} 259 nm, 10.4 OD259 units (corresponding to a total of 130 OD259 units or 1.56 µmoles, assuming 84,000 for ϵ for this octamer). Therefore the overall yield through the seven cycles in synthesis, deprotection, cleavage from the silica support, and isolation amounts to 31% based on thymidine initially bound to silica. This material was degraded by snake venom phosphodriesterase to the component nucleotides.

Chromatograms of the crude mixture obtained directly from the solid phase support, and of the product isolated after TLC on silica, are shown in Figure 1. Better resolution was achieved on the reverse phase column than on the ion change column in this case. It may be noted that the octanucleotide was the major component in the synthetic mixture. Extensive purification was achieved by the TLC separation, but a small amount of con-

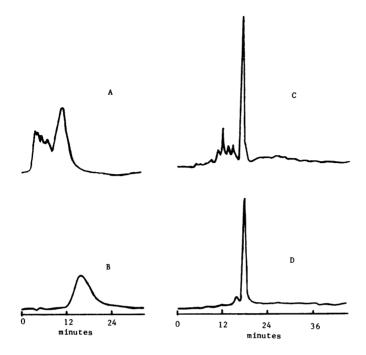


Figure 1. HPLC of reaction mixture from synthesis of d-TGCAGGTT. Flow rate 1 mL/min. A and B: Whatman PXS 10/25 SAX column. A, crude reaction mixture obtained on cleaving products from solid support; solvent 0.3 M triethylamine acid 0.15 n citric acid in water. B. Product after separation on silica plate; 0.2 M triethylamine and 0.1 M citric acid. C and D, Whatman Partisil PX10/25 ODS-2 column; gradient, 10% to 19% acetonitrile in 0.1 M aqueous triethylammonium acetate; A, crude product obtained from solid support; B, product after isolation on silica gel.

taminant remained (see D in Figure 1). Preparative HPLC of the product from silica gel chromatography on the reverse phase column afforded good quality octanucleotide, which ran as a single peak on HPLC. This material on phosphorylation with $[\gamma-3^2P]$ -ATP and polynucleotide kinase and electrophoresis on a polyacrylamide gel migrated as an octanucleoside heptaphosphate. The sequence was shown to be d-TGCAGGTT by a modification of the Maxam-Gilbert procedure for sequencing DNA.

<u>Variables in the Synthetic Sequence</u>. In seeking to optimize conditions for routine syntheses we examined the individual steps (de-dimethoxytritylation, condensation, oxidation, capping, and cleavage) with substrates bound covalently to the silica support.

De-dimethoxytritylation. One of the troublesome steps in chemical synthesis of oligonucleotides is deprotection of the terminal 5'-O groups.

Schaller and Khorana⁸ first noted that acidic conditions for removing dimethoxytrityl protecting groups can lead to extensive loss of N-benzoyl-adenine from deoxyadenosine derivatives. In recent years benzenesulfonic acid⁹ and trichloroacetic acid¹⁰ in chloroform have been widely used for this purpose even though some depurination is known to take place. The most selective reagent is zinc bromide; indeed, no depurination was observed in model studies conducted over a period of 24 hours.² For rapid syntheses, however, zinc bromide suffers the disadvantage of a slow rate of reaction with substances bound to silica supports (30 minutes or more required).

Results of a survey of reactions of various acidic reagents with N-benzoyldeoxyadenosine derivatives are presented in Table II. Several conclusions may be drawn from the data. (1) A 5'-terminal d-bzA group is more sensitive to acid than an internal d-bzA group (i.e., one flanked by a nucleoside triester at the 5'-0.11 (2) The effect of methanol in nitromethane, which retards depurination, is pronounced even at a 1% methanol level. (3) Trichloroacetic acid (3%) in nitromethane/methanol (99/1 v/v)

Table II. Depurination of Substrates Bound to Silica

Reagent	% Depurination of Bound Substrate in 1 hour			
	d-(DMTr)bzA-	d-(DMTr)bzApT-	d-(DMTr)Tpbz	A- d-(DMTr)ibG-
ZnBr ₂ in CH ₃ NO ₂	[0]	[0]	[0]	[0]
2% C ₆ H ₅ SO ₃ H in	72	50	18	7
Сн ₂ С1 ₂ /Сн ₃ Он 7/3				
3% Cl ₃ CCOOH in	67	47	12	7
CH ₂ Cl ₂				
3% Cl ₃ CCOOH in CH ₃ NO	2 49	26	6	4
3% Cl3CCOOH in CH3NO	2/			
Сн ₃ он 95/5	11	15	3	2
98/2	16			
99/1	18			

The sample (~40 mg) with nucleoside or nucleotide bound to silica was treated with the acidic reagent (1 mL, 60 min, room temperature), washed well with THF then ether, dried, and treated with NH₄OH (50°C, 15 h). The solutions were lyophilized and the nucleotide taken up in H₂O. The extent of depurination was calculated from observed absorbance values (A₂₆₁ for dA derivatives, A₂₅₂ for dG), and extinction coefficients for dA, dT and benzamide, relative to the absorbance for products from the ZnBr₂ reactions, for which depurination is taken as zero. (In the symbols for the silicabound nucleotides <u>p</u> refers to an internucleotide methyl phosphotriester group.)

is an effective reagent for the de-dimethoxytritylation step. With this reagent, cleavage of the dimethoxytrityl ethers is complete in less than three minutes. In this time period depurination of a terminal d-bzA is negligible. Furthermore, the internal d-bzA units, which are exposed to multiple acid treatments in the course of a synthesis, show good stability even over relatively long periods (about 3% degradation in 60 minutes).

For further information d-(DMTr)bzApTa SiO₂ and d-(DMTr)TpbzA SiO₂ were synthesized as described in Tables 1 and 2; then the samples were treated with the acidic reagents (Figure 2) for 1 h and the nucleotidic materials were recovered by conventional treatment with C₆H₅SH/(C₂H₅)₃N/dioxane followed by NH₄OH (15 h). Chromatographic profiles for the reaction products (d-ApT plus small amounts of degradation materials) obtained from d-(DMTr)bzApTa SiO₂ are shown in Figure 2. These experiments confirm the conclusions drawn from data in Table II; namely, that some side products are formed on extended exposure of silica bound d-bzA derivatives to CCl₃COOH in CH₂Cl₂ or 2% benzenesulfonic acid in CH₂Cl₂/CH₃OH 7/3, but very little if any degradation takes place when de-dimethoxytritylation is achieved with ZnBr₂ in CH₃NO₂ or CCl₃COOH in CH₃NO₂ plus a little CH₃OH. The experiments with d-(DMTr)TpbzA~SiO₂ gave the same order of reactivity but showed much less degradation under the acidic conditions.

Condensation Reaction. The extent of reaction of the 5'-OH of a terminal bound nucleoside with a nucleoside phosphorochloridite reagent can be measured by the amount of dimethoxytrityl cation liberated in a subsequent acid cleavage step. We found by this test that 6 minutes was sufficient time for the condensation step in synthesizing oligomers on the silica support. Yields for reactions of derivatives of each of the common nucleosides averaged above 90% per cycle (Table III). With shorter reaction times the conversions were lower (average of 70% and 89% per cycle in constructing d-(Tp)5T with 2 minute and 3 minute reaction times, respectively) and with longer reaction times the yields were not improved.

Oxidation. For estimation of the rate of oxidation on the silica support, a sample of d-(DMTr)TpT \sim (\widetilde{SiO}_2) was prepared and exposed to I₂ (0.1 M I₂ in THF/pyridine/water 40/20/1 v/v/v) for periods of 0,10,30,60 and 180 seconds, following which the solid was washed and treated with ammonium hydroxide (50°C for 15 h) and the d-TpT was recovered by chromatography on Avicel pates (phosphites are hydrolyzed under the ammoniacal conditions; no dTpT was obtained from the 0 time reaction). This experiment showed the oxidation to be very fast, being complete within 10 seconds. As a safety

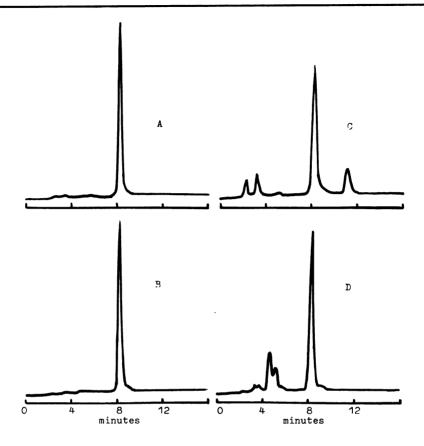


Figure 2. a-(DMTr)bzApT~(\$10) was prepared by the syringe technique. Samples of the silica derivative were treated for 1 h with A, ZnBr2 in CH3NO2; B, 3% CCl3CCOOH in CH3NO2/CH3OH 95/5; C, 3% CCl3COOH in CH2Cl2; and D, 2% C6H5SO3H in CH2Cl2/CH3OH 7/3. The samples were then washed and worked up by treatment with C6H5SH followed by NH4OH as usual. The nucleotide products were analyzed by HPLC without further purification, using a Whatman Partisil PXIO/25 ODS-2 column with a gradient of 10% acetonitrile and 0.1 M aqueous triethylammonium acetate, increasing acetonitrile at the rate of 0.2%/min; flow rate, 1 mL/min.

factor 30 seconds is recommended for routine oxidations using the syringe technique.

m-Chloroperbenzoic acid in methylene chloride has been suggested as an oxidant in place of iodine-water since it reacts rapidly and quantitatively with phosphites under anhydrous conditions. 12 We have found that although this reagent is suitable for use with dT and d-ibG derivatives, it attacks d-bzC rapidly and d-bzA slowly, both in solution and in solid phase syntheses. On the other hand, m-chloroperbenzoic acid can be used safely

Table III	. %	Conversion	in	Chain	Elongation

Synthetic Product	Average % Conversion per Cycle in Synthesis
d-T ₆	98
d-T9	96
d-(ibG) ₅ T	92
d-(bzA) ₆ T	91
d-(bzC) ₆ T	95

Syntheses were carried out on silica gel by the syringe technique. The numbers are averages of conversions through the synthesis as measured by A_{500} for the dimethoxytrityl cation liberated on acid cleavage.

if pyridine is present. With a solution of 0.1 M m-chloroperbenzoic acid in methylene chloride/pyridine (10/1 v/v) silica bound phosphites are oxidized rapidly (<30 seconds) without attack at d-bzC or d-bzA residues. It is essential that pyridine be added just prior to use since the reagent loses its oxidizing potential within 15 minutes after pyridine is added.

Capping. Procedures for synthesizing oligonucleotides on solid supports generally include a "capping" step to block any 5'-OH groups that failed to react with the phosphoryl or phosphite reagent. Several experiments in our laboratory indicated that the capping step may not be necessary in reactions involving excess nucleoside chloridites, and that failure to achieve quantitative conversions stems from causes other than failure of the hydroxyl groups to react. The synthesis of the octanucleotide described here indeed shows that the capping step was not essential for this sequence. If a capping step is desired, however, it can be included without much loss in time since acetylation by acetic anhydride in the presence of p-dimethylaminopyridine3 is very fast. In model experiments the extent of acetylation of d-bzC~(SiO2) (31 mg) with 1 mL of acetic anhydride/pyridine/dimethylaminopyridine (0.5 mL/4.5 mL/30 mg) was monitored by subsequent condensation with d-(DMTr)TmCl, oxidation, and acid cleavage of the dimethoxytrityl group as in the synthetic cycle. This experiment showed that the acetylation reaction was over within 30 seconds under these conditions.

Cleavage from the Silica Support. Two procedures have been described for cleaving oligomers from the silica support. One (I) involves initial treatment with thiophenol and triethylamine (to deprotect the phosphotriesters) followed by ammonium hydroxide (to sever the anchor link).² In the other (II) the silica product is treated directly with ammonium

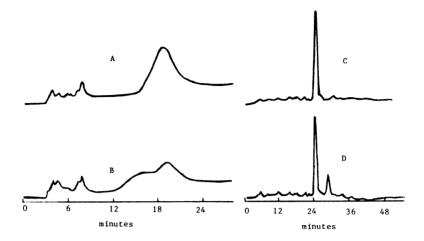


Figure 3. HPLC of crude reaction mixture from synthesis of d-(Tp)₆T; flow rate 1 mL/min. A and C: products cleaved by method I (C₆H₅SH then NH₄OH). B and D: products cleaved by method II (NH₄OH). A and B: Whatman PXS 10/25 SAX column; gradient 0.125 M triethylamine-0.075M citric acid to 0.25 M triethylamine -0.15 M citric acid. C and D: Whatman Partisil PX 10/25 ODS-2 column; gradient 10% to 15% acetonitrile in 0.1 M aqueous triethylammonium acetate.

hydroxide, wherein both P-OCH₃ deprotection and cleavage of the ester link anchoring the oligomer are brought about by the same reagent.³ We have used both procedures in isolating d-(Tp)₆T in a given synthesis. Chromatography of nucleotide mixtures eluted from the silica samples gave the profiles shown in Figure 3. A significant side product was found in the material recovered by procedure II which was not present in material obtained by procedure I. This material has not been characterized; however since it migrates faster on the anion exchange column, and slower on the reverse

Table IV. Synthesis of d-(Tp)6T

Oxidizing Agent	Cleavage Agent	Yield of Isolated Nucleotide
I ₂ -н ₂ о	I. C ₆ H ₅ SH, then NH ₄ OH	57
	II. NH ₄ OH only	41
<u>т</u> -с1с ₆ н ₄ со ₃ н	I. C ₆ H ₅ SH, then NH ₄ OH	55
	II. NH4OH only	32

Standard conditions were used with variations indicated. The yield represents product recovered after chromatography on silica plates, relative to the initial thymidine unit bound to the solid support.

phase column, than d-(Tp)6T, it seems likely that it is a derivative of the heptanucleotide in which one of the internucleoside links is a phosphoramidate (-OP(O)(NH₂)O-, formed by nucleophilic attack of NH₃ on the phosphotriester) rather than a phosphodiester (-OP(O)(O⁻)O-). On isolation of the heptanucleotide by chromatography on silica gel, a 57% yield of d-(Tp)6T was obtained for the sample worked up by procedure I, but only 41% was recovered as d-(Tp)6T for that worked up by procedure II (Table IV). A similar result was obtained for a synthetic sequence in which oxidation in each cycle was achieved with m-chloroperbenzoic acid/pyridine rather than iodine/water. It is therefore clear both from the chromatographic profiles and the yields of recovered product that the thiophenol step serves a useful purpose and should be included in the protocol.

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